SUPPLEMENTARY APPENDIX

Dose-adjusted EPOCH and rituximab for the treatment of double expressor and double-hit diffuse large B-cell lymphoma: impact of *TP53* mutations on clinical outcome

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Supplementary

Methods

Immunohistochemistry and Fluorescence in situ hybridization analysis

All steps were performed at room temperature. 3-3'-Diaminobenzidine tetrahydrochloride (DAB) was incubated for 10 min as a chromogen, and Mayer's hematoxylin was used for counterstaining for 10 min. The slides were dehydrated, cleared, and mounted with coverslips. We used CD10, BCL-6 , and MUM1 staining to divide all DLBCL cases into GCB or non-GCB subgroups according to the Hans[1].

Cases were considered positive for MYC if ≥40% of tumor cells were stained with the antibody. A cut-off level of 50% positive cells was used for BCL-2[2]. The cut-off values for BCL-6, MUM-1, and CD10 were set at 30%, according to recent literature. For FISH analyses, at least 100 nuclei were counted. Rearrangement was defined as the presence of break-apart signals in ≥15% of nuclei. DH and TH lymphomas were defined as the concurrent rearrangement of MYC and BCL2 and/or BCL6[3].

Sanger Sequencing

We performed the analysis of the TP53 mutation profile on FFPE specimens, using the Maxwell RSC, Promega for DNAs extraction ((Promega Corporation, Madison, Wisconsin, USA). Only a few biopsies were available as fresh tissues. Sanger's direct sequencing method was used for fresh biopsies. The analysis included coding sequences from exon 4 to exon 10, the most frequently involved regions). The cutoff for positivity of the mutation status was 20% of examined alleles, that is 10% of all cells. In order to establish the pathogenic role of our findings, we compared these results with the International Agency for Research on Cancer (IARC) TP53 mutation database. All the not pathogenic variants and silent mutations were considered as wild type.

Next-Generation Sequencing

TP53 mutations were analyzed using the Ion AmpliSeq™ TP53 Panel (Thermo Fisher Scientific, Inc, Waltham, Massachusetts, USA) designed to investigate all coding exons of

TP53 with 24 amplicons. Briefly, 40 ng of DNA extracted from FFPE diagnostic tissues were amplified, fragmented, ligated to adapters, barcoded, and clonally amplified onto beads to create DNA libraries. Following quality control analysis and quantification by the 4200 TapeStation System (Agilent Technologies, Inc., Santa Clara, CA, USA), library mixtures were amplified and enriched. Finally, the library pool was sequenced with the Ion PGM™ Hi-Q™ sequencing kit (Thermo Fisher Scientific, Inc.). The mutation sites were analyzed by the IonTorrent variant caller plugin v5.12 according to the reference genome hg19 and the IARC TP53 database: http://p53.iarc.fr/TP53GeneVariations.aspx or TP53 web site: http://p53.fr/ and data reported as suggested. Identified mutations were confirmed by direct sequencing.

Follow-up

An intermediate disease assessment using CT was performed after 3 or 4 cycles of DA-EPOCH-R. Patients who exhibited less than partial response (PR) or progression of disease (PD) were shifted to second-line regimens according to institutional guidelines. Evaluation of final clinical response was performed at the end of cycle 6 using CT, PET , and bone marrow biopsy, when the biopsy was positive at disease onset. Disease assessment was performed during follow-up at 3-month intervals for the first 2 years, every six months until the 5th year, and annually thereafter. Response evaluation was assessed using the Lugano Revised Response Criteria[4].

Statistical Analyses

Progression-free Survival was defined as the time interval between diagnosis and disease progression or death due to any cause, whichever occurred first. Time was censored at the latest follow-up for living patients who were progression-free. Overall Survival was defined as the time interval between diagnosis to death due to any cause. Time was censored at the latest follow-up for living patients. The OS and PFS curves were estimated using the Kaplan-Meier method and the curves were compared using the log-rank test. Crude cumulative incidence of CNS relapse was estimated in a competing risk setting using cumulative incidence estimates and death without relapse was evaluated as a competing event. The

median follow-up was estimated with the reverse Kaplan–Meier method using OS data. All reported P values were two-sided.

Univariable and multivariable Cox models were performed to assess the association between the main patients and disease characteristics and the outcomes. Multivariable models included the statistically significant variables at univariable analysis.

References

1) Hans CP, Weisenburger DD, Greiner TC, et al. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood. 2004; 103 (1): 275-283.
2) Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization (WHO) classification of lymphoid neoplasms. Blood. 2016; 127(20): 2375-2390.
3) Horn H, Ziepert M, Becher C, et al. MYC status in concert with BCL2 and BCL6 expression predicts outcome in diffuse large B-cell lymphoma. Blood. 2013; 121(12):2253-2263.
4) Cheson BD, Fisher RI, Barrington SF, et al. Recommendations for Initial Evaluation, Staging and Response Assessment of Hodgkin and Non-Hodgkin Lymphoma: The Lugano Classification. J Clin Oncol. 2014 : 32 (27): 3059-3067.

Tables

Supplementary Table 1. Clinical Characteristics of 27 patients with limited disease at presentation

	N=27
Age, continuous (years)	
Median (third and first quartile)	57.00 (45.00; 69.50)
Rearrangements	
DEL only	18 (66.7)
DEL-BCL2	2 (7.4)
DEL-MYC	4 (14.8)
DEL-DH/TH	3 (11.1)
Ki67 (%)*	
Median (third and first quartile)	87.50 (77.50; 90.00)
Sex	
Male	17 (63.0)
Female	10 (37.0)
Cell of origin	
Germinal central B-cell	13 (48.1)
Non-Germinal central B-cell	13 (48.1)
Not assessed	1 (3.7)
CNS-International prognostic index	
0-1	23 (85.2)
2-3	4 (14.8)
4-6	0 (0.0)
Systemic CNS therapy	
None	2 (7.4)
Intrathecal methotrexate	7 (25.9)
Intravenous methotrexate	18 (66.7)

Abbreviations: DH/TH, double/triple hit; CNS, central nervous system.
*3 missing values

Supplementary Table 2. Clinical Characteristics of 22 patients underwent Autologous Stem-cell Transplantation

	N=22
Age, continuous (years)	
Median (third and first quartile)	59.00 (49.00; 62.00)
Age, categorical (years)	
≤60	13 (59.1)
>60	9 (40.9)
Rearrangements	
DEL only	12 (54.5)
DEL-BCL2	3 (13.6)
DEL-MYC	1 (4.5)
DEL-DH/TH	6 (27.3)
Ki67 (%)*	
Median (third and first quartile)	80.00 (70.00; 86.25)
Sex	
Male	10 (45.5)
Female	12 (54.5)
Cell of origin	
Germinal central B-cell	12 (54.5)
Non-Germinal central B-cell	7 (31.8)
Not assessed	3 (13.6)
Staging	
I-II	3 (13.6)
III-IV	19 (86.4)
International prognostic index	
0-2	7 (31.8)
3-5	15 (68.2)
CNS-International prognostic index	
0-1	1 (4.5)
2-3	13 (59.1)
4-6	8 (36.4)
Extranodal sites risk CNS	
Yes	3 (13.6)
No	19 (86.4)

Abbreviations: DH/TH, double/triple hit; CNS, central nervous system.

*2 missing values

Supplementary Table 3. Clinical characteristics of 122 consecutive Diffuse large B-cell lymphoma patients according to sex

	Male N=75	Female N=47
Age, continuous (years)		
Median (third and first quartile)	61.00 (55.00; 65.00)	56.00 (43.00; 63.50)
Age, categorical (years)		
≤60	36 (48.0)	30 (63.8)
>60	39 (52.0)	17 (36.2)
Rearrangements		
DEL only	49 (65.3)	32 (68.1)
DEL-BCL2	7 (9.3)	6 (12.8)
DEL-MYC	6 (8.0)	3 (6.4)
DEL-DH/TH	13 (17.3)	6 (12.8)
Ki67 (%)*		
Median (third and first quartile)	90.00 (72.50; 92.50)	85.00 (80.00; 90.00)
Cell of origin		
Germinal central B-cell	35 (46.7)	20 (42.6)
Non-Germinal central B-cell	36 (48.0)	24 (51.1)
Not assessed	4 (5.3)	3 (6.4)
Staging		
I-II	17 (22.7)	10 (21.3)
III-IV	58 (77.3)	37 (78.7)
International prognostic index		
0-2	34 (45.3)	21 (44.7)
3-5	41 (54.7)	26 (55.3)
CNS-International prognostic index		
0-1	21 (28.0)	10 (21.3)
2-3	36 (48.0)	25 (53.2)
4-6	18 (24.0)	12 (25.5)
Extranodal sites risk CNS		
Yes	7 (9.3)	9 (19.1)
No	68 (90.7)	38 (80.9)
CNS prophylaxis		
None	10 (13.3)	6 (12.8)
Intrathecal methotrexate	25 (33.3)	15 (31.9)
Intravenous methotrexate	40 (53.3)	26 (55.3)
Autologous stem-cell transplantation		
Yes	10 (13.3)	12 (25.5)
No	65 (86.7)	35 (74.5)

Abbreviations: DH/TH, double/triple hit; CNS, central nervous system.

*10 missing values: 8 in male and 2 in female group

Supplementary Table 4. Results of the Univaraible Cox models for Progression-free and Overall Survival according to patients and disease characteristics

	Progression-Free survival		Overall survival	
	Hazard ratio (95% CI)	P*	Hazard ratio (95% CI)	P*
Age (continuous)		0,129		0,205
Linear	1.51 (0.89; 2.57)		2.39 (0.87; 6.54)**	
Age		0,143		0,073
>60 vs ≤60	1.75 (0.83; 3.70)		2.35 (0.92; 5.99)	
Sex		0,018		0,105
Female vs Male	0.31 (0.12; 0.81)		0.40 (0.13; 1.21)	
Cell of origin***		0,545		0,905
GCB vs non-GCB	1.26 (0.60; 2.64)		0.95 (0.38; 2.33)	
Rearrangements****		0,188		0,124
DEL-BCL2 vs DEL	1.69 (0.523; 4.38)		0.64 (0.07; 2.67)	
DEL-MYC vs DEL	0.23 (0.00; 1.66)		0.32 (0.00; 2.46)	
DEL-DH/TH vs DEL	1.83 (0.73; 4.17)		2.51 (0.95; 6.23)	
Staging		0,067		0,117
III-IV vs I-II	3.84 (0.91; 16.14)		5.00 (0.67; 37.48)	
International prognostic index		0,004		0,007
3-5 vs 0-2	3.71 (1.50; 9.14)		5.48 (1.59; 18.81)	
Systemic CNS therapy		0,037		0,019
None vs Intravenous MTX	3.61 (1.31; 9.96)		6.47 (1.73; 24.17)	
Intrathecal MTX vs Intravenous MTX	2.08 (0.90; 4.81)		3.56 (1.11; 11.36)	
TP53 mutation*****		0,042		0,049
Mutated vs Wild type	2.93 (1.04; 8.25)		3.30 (1.01; 10.82)	

Abbreviations: CI, confidence interval; GCB, Germinal central B-cell; DEL, double expressor lymphomas; DH/TH, double-hit/triple-hit; CNS, central nervous system, MTX, methotrexate.

*Wald test p-value

**Modeled as restricted cubic spline and reporteing result of 65 vs 49 years comparison

***Excluding 7 not-assessed patients

****Performed with Firth's penalized maximum likelihood bias reduction method

*****Excluding 53 not-assessed patients

Supplementary Table 5. Clinical characteristics of 122 consecutive Diffuse large B-cell lymphoma patients according to central nervous system prophylaxis

	None N=16	Intrathecal MTX N=40	Intravenous MTX N=66
Age, continuous (years)			
Median (third and first quartile)	65.00 (61.25; 72.00)	57.50 (48.75; 63.00)	57.00 (46.25; 64.00)
Age, categorical (years)			
≤60	4 (25.0)	23 (57.5)	39 (59.1)
>60	12 (75.0)	17 (42.5)	27 (40.9)
Rearrangements			
None	12 (75.0)	24 (60.0)	45 (68.2)
DEL-BCL2	0 (0.0)	5 (12.5)	8 (12.1)
DEL-MYC	1 (6.2)	3 (7.5)	5 (7.6)
DEL-DH/TH	3 (18.8)	8 (20.0)	8 (12.1)
Ki67 (%)*			
Median (third and first quartile)	90.00 (82.50; 90.00)	90.00 (75.00; 95.00)	85.00 (77.50; 90.00)
Sex			
Male	10 (62.5)	25 (62.5)	40 (60.6)
Female	6 (37.5)	15 (37.5)	26 (39.4)
Cell of origin			
Germinal central B-cell	7 (43.8)	20 (50.0)	28 (42.4)
Non-Germinal central B-cell	8 (50.0)	17 (42.5)	35 (53.0)
Not assessed	1 (6.2)	3 (7.5)	3 (4.5)
Staging			
I-II	2 (12.5)	7 (17.5)	18 (27.3)
III-IV	14 (87.5)	33 (82.5)	48 (72.7)
International prognostic index			
0-2	5 (31.2)	15 (37.5)	35 (53.0)
3-5	11 (68.8)	25 (62.5)	31 (47.0)
CNS-International prognostic index			
0-1	1 (6.2)	8 (20.0)	22 (33.3)
2-3	9 (56.2)	19 (47.5)	33 (50.0)
4-6	6 (37.5)	13 (32.5)	11 (16.7)
Extranodal sites risk CNS			
Yes	0 (0.0)	5 (12.5)	11 (16.7)
No	16 (100.0)	35 (87.5)	55 (83.3)
Autologous stem-cell transplantation			
Yes	0 (0.0)	8 (20.0)	14 (21.2)
No	16 (100.0)	32 (80.0)	52 (78.8)

Abbreviations: MTX, Methotrexate; DH/TH, double/triple hit; CNS, central nervous system.

**10 missing values: 1, 2, and 7 not treated, intrathecal methotrexate, and intravenous methotrexate group, respectively*

Supplementary Figure 1: Progression-Free Survival (A) and Overall Survival (B) in patients who underwent or not Autologous Stem Cell Transplantation.